In The United States Patent and Trademark Office

In re U.S. Patent Application of: **Zsuzsanna NAGY**	Examiner: **Burkhart, Michael D.**
Serial No. **10/659,578**	Group Art Unit: **1633**
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For: **Diagnostic Screens for Alzheimer's Disease**	Confirmation No. **4669**

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Declaration of Zsuzsanna Nagy, Ph.D. Pursuant to 37 C.F.R. §1.132

Sir:

I, **Zsuzsanna Nagy**, **Ph.D.**, hereby declare as follows:

1. I believe that I am the original and sole inventor of the subject matter which is claimed and for which a patent is sought on the above-captioned patent application, the specification of which was filed on September 10, 2003, as Application Serial No. 10/659,578 (the "Application").

2. I have reviewed, and am familiar with, the Application (including its pending claims), and the Office Action of the United States Patent and Trademark Office (the "Office") mailed on March 6, 2007 (the "Office Action") with respect to the Application.

3. I am currently employed as a University Lecturer with the University of Birmingham Medical School in Birmingham, UK, where I conduct research in the pathogenesis of neurodegenerative disorders. I received my D.Phil. in Physiological Sciences from the University of Oxford (UK) in 1995, and was a post-doctoral fellow at the Oxford Project to Investigate Memory and Ageing (OPTIMA) from 1995 to 2004. I completed my medical training in 1983-1989. I have a business relationship with the licensee of the present application.

4. I have over 15 years experience in medicine, molecular biology, neurodegeneration and the study of neurodegenerative disorders, including cell cycle regulation. I am familiar with experimental design, cell culture systems, animal models, clinical diagnostic and treatment methods for neurodegenerative disorders including dementias, and biochemical and molecular biology laboratory techniques. I am therefore qualified to opine as to the enablement of those of ordinary skill to practice the invention presently being claimed in the Application.

5. I have made this Declaration in order to provide evidence addressing the following points:

(a) the Office's arguments regarding a publication I authored, entitled "The Dysregulation of the Cell Cycle and the Diagnosis of Alzheimer's Disease", *Biochimica et Biophysica Acta* 1772:402-408 (2006 online; 2007 in print) (the "2006 Publication");

(b) the Office's arguments that the Application reports experimental data contradictory to that found in a publication I co-authored, entitled "Cell Cycle Kinesis in Lymphocytes in the Diagnosis of Alzheimer's Disease", Neuroscience Letters 317:81-84 (2002) (the "2002 Publication");

(c) the Office's arguments that the Application fails to provide those of ordinary skill with sufficient guidance to enable the diagnosis of Alzheimer's Disease (AD) as opposed to cancer by determining the effectiveness of the G1/S cell cycle checkpoint control in non-neuronal cells; and

(d) the Office's arguments with respect to the ability of the ionizing and UV radiation to induce G1 cell cycle arrest.

The 2006 Publication

6. The Office Action argues that the claimed methods are unpredictable, because the 2006 Publication "teaches that diagnosis of 'definite' Alzheimer's can only be made by histopathological assessment after autopsy, and that clinical diagnostic criteria (NINCDS-ARDRA) have a very high false negative rate." Office Action at 3.

7. I respectfully disagree with the Office's reasoning, because the ability of the claimed methods to distinguish, for example, Alzheimer's Disease (AD) patients from normal controls, and thereby diagnose AD, is supported by experimental data. Moreover, no diagnostic method is capable of 100% accuracy, and the claimed methods for diagnosing neurodegenerative disease should not be held to such a high standard.

8. The 2006 Publication does contain a statement that "definite" diagnosis of AD can only be performed after patient death, but such an absolutely perfect diagnosis is not useful in a medical sense because it does not enable a *live* patient with AD to be diagnosed *in time to be treated*. Diagnostic practitioners often rely on diagnostic methods and criteria that are not perfect, or that yield false positives or false negatives, because such methods are nevertheless still very useful in diagnosis. For example, the NINCDS-ARDRA criteria are widely used for diagnosing AD, because they are very specific for positive results, in that a NINCDS-ARDRA diagnosis of AD has a 98-100% positive predictive value when followed by post-mortem examination. The false negative rate of the NINCDS-ARDRA criteria does not detract from their usefulness as a diagnostic tool for the identification of AD patients.

9. Moreover, the experiments described in the Application report a correlation between the presence of G1/S cell cycle checkpoint regulatory defects in subjects diagnosed with AD as assessed by the NINCDS-ARDRA criteria and preAD as assessed by neuropsychological testing. Because the NINCDS-ARDRA criteria have such a high positive predictive value (98-100%), a diagnostic practitioner skilled in the art would infer that the checkpoint regulatory defects found in the Application's experimental subjects can also be relied upon as 98-100% specific for AD as determined post-mortem. This is a very high positive predictive value, and underscores the usefulness of the claimed methods in diagnosing neurodegenerative disease in patients prior to their death.

10. Therefore, because the claimed methods have a positive predictive value at least as good as the NINCDS-ARDRA criteria, diagnostic practitioner skilled in the art would recognize their ability to reliably diagnose a patient with AD.

No Discrepancy Exists Between The Application And The 2002 Publication

11. The Office Action compares Fig. 1A of the 2002 Publication with Figure 2 of the Application, states that these experimental results are different, and argues that "this discrepancy calls into question the reliability and predictability of the instantly claimed methods." Office Action at 3-4.

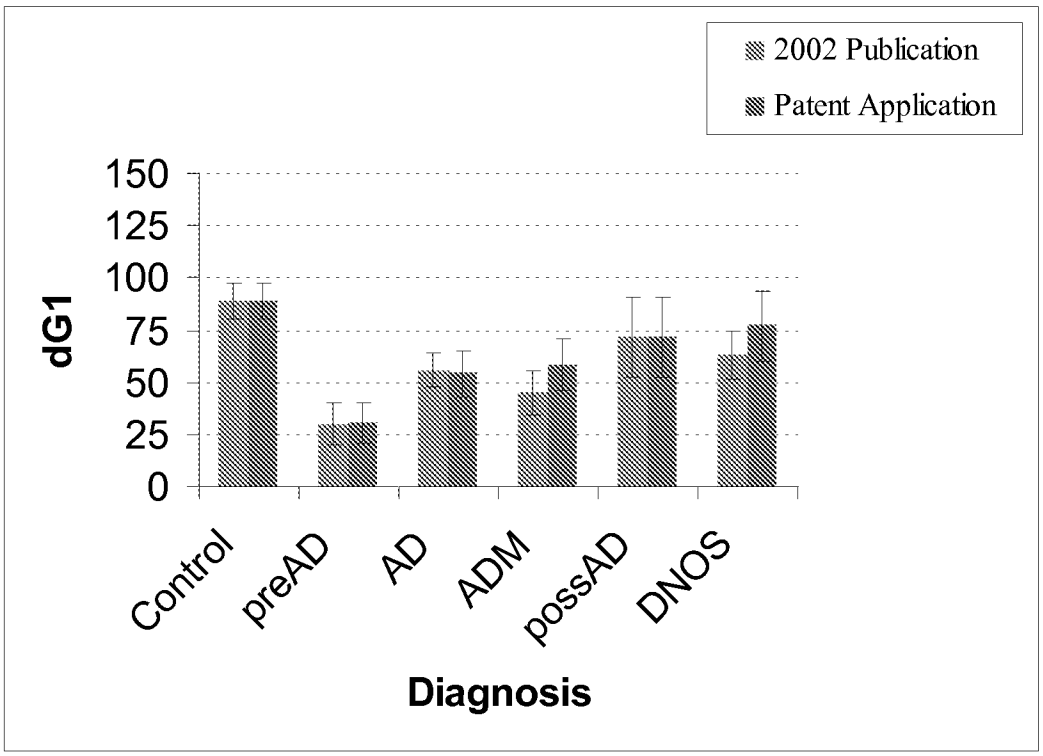
12. It is my opinion that there is no discrepancy between the data reported in the Application and the data reported in the 2002 Publication. Although there are some minor variations in the results reported, both of these documents report significant differences in the relative G1 lengthening between the Alzheimer's groups (preAD, AD, ADM) and the other subject groups.

13. The Application discloses an experiment in Example 1 that involved screening for a cell cycle regulatory defect (e.g., the effectiveness of the G1/S cell cycle checkpoint control) by measuring the relative lengthening of the G1 phase of the cell cycle when exposed to a cell division inhibitor. *See* Application at page 23, line 14 through page 27, line 32. The experiment was performed on a total of 49 subjects, including 14 controls. *Id.* at Table 1a. The results of this experiment, which are shown in Figure 2 of the application, report significant differences between the Alzheimer's groups (preAD, AD, ADM) and the other subject groups, in both the raw and age-corrected data. In the 2002 Publication, I describe an experiment similar to that provided in the Application, but involving a total of 66 subjects, including 15 controls. *See* 2002 Publication at 82 (Table 1a). The results of this experiment, which shown in Figure 1A of the 2002 Publication, also reveal significant differences between the Alzheimer's groups (preAD, AD, ADM), which had low values, and the other subject groups, which had high values.

14. It is thus seen that the experiments in the Application and the 2002 Publication yield consistent experimental results. In particular, with respect to other neurodegenerative diseases (the possAD and DNOS groups relative to the control), the Application and the 2002 Publication both report similar results, i.e., the control group has the highest value, followed by the possAD group, and then the DNOS group. *Compare* Application Figure 2 (left-hand chart) *with* 2002 Publication Figure 1A.

15. Likewise, with respect to the AD and ADM groups relative to each other, the Application and the 2002 Publication both yield similar data for the relative lengthening of the AD and ADM groups. The reported difference in the relative scores of the AD and ADM groups between the two experiments is most likely the result of normal experimental variance (all diagnostic tests have variance, the normal values always have a lower and upper limit), and the differing sample size between the two experiments (the Application involved 9 AD and 7 ADM subjects, whereas the 2002 Publication involved 17 AD and 10 ADM subjects). Moreover, the relative scores of the AD and ADM groups are not relevant to the claimed methods, which rely on the comparison between the tested subject against a control to diagnose the respective neurodegenerative disease, and not on a comparison between tested subjects.

Figure 1. Comparison of data included in the Patent application and the 2002 Publication. Differences between mean values are significant ONLY if the error bars DO NOT overlap. The lack of significant differences between the two sets of data indicates a good reproducibility of the test.



16. I therefore respectfully disagree with the Office Action, because it is my opinion that the Application and the 2002 Publication report experimental results that, while not identical, are highly consistent, and thus demonstrate the reliability and predictability of the use of relative G1 lengthening as a diagnostic for neurodegenerative disease.

The Application Provides Sufficient Guidance To Those Of Ordinary Skill

17. The Office Action states that "the instantly claimed methods and specification ignore the fact that merely assaying for a defect in the G1/S checkpoint, or relative resistance to the effects of a G1 inhibitor such as rapamycin, then diagnosing patients with such a G1/S defect as having AD would misdiagnose many cancer patients as having AD. Chan (Brit. J. Canc., 2004) and Wendel et al (Nature, 2004) document cancer cells resistant to rapamycin. Absent evidence to the contrary, testing these rapamycin-resistant cells using the instantly claimed methods would produce results similar to those seen for the AD cells, i.e., a resistance to the effects of rapamycin." Office Action at 4-5.

18. It is my opinion that the instantly claimed methods would distinguish between the diagnosis of AD and cancer, for several reasons:

A. First, the present independent claims recite a diagnostic method *comprising* determining the effectiveness of the G1/S cell cycle checkpoint, and thus do not exclude additional steps from their scope. Based on my experience in medicine and clinical diagnostics, it is my opinion that a diagnostic practitioner skilled in the art would not necessarily rely upon a single test result as conclusive of a diagnosis, but instead would apply her training and judgment in interpreting the test result in the context of the patient's overall medical profile. Thus, like any other diagnostic method, the claimed method enables (but does not mandate) the diagnostic practitioner skilled in the art to make a diagnostic conclusion based on the results of the method. For example, a diagnostic practitioner skilled in the art would not suspect a patient of having AD instead of cancer if the patient exhibited abnormal blood test readings typical of a cancer (e.g., abnormal sedimentation

rate or complete blood count (CBC), etc.) in addition to a positive result from the claimed methods.

B. Second, the diagnostic capability of the claimed methods is based on findings that the dysregulation of the cell cycle in AD is reflected not only in the diseased cells (neurons) themselves, but also in non-neuronal cells such as lymphocytes and fibroblasts. Thus, diseased neurons may be diagnosed by performing tests on non-neuronal cells. In contrast, the Chan and Wendel et al. articles cited by the Office have found that certain cancer cells may themselves exhibit resistance to rapamycin, but have not shown that such resistance is exhibited in *other* cells within the subject's body. Thus, even assuming that the Office's conjecture was correct, the claimed methods would reveal a "false" diagnosis of AD in a subject with cancer only if the cancer cells themselves were the tested cells of the claimed methods. This situation, however, would not occur because a diagnostic practitioner skilled in the art is able to distinguish cancerous cells from non-cancerous cells, and would typically seek to extract and employ the far more numerous and far more accessible non-cancerous cells of the patient rather than conduct a far more exacting tumor biopsy in performing the claimed methods. Thus, there is little likelihood of misdiagnosing a subject due to the subject having cancer. I note in addition that the longevity of cancer patients is unfortunately typically *decades* shorter than that of a patient having a neurodegenerative disease (e.g., AD). Thus, there would seem to be no basis for supposing that a cancer patient would seek a diagnosis of a long term neurodegenerative disease, or that a physician would conduct such an analysis for the patient. Thus, I submit that the Office's concern is misplaced.

19. I therefore respectfully disagree with the Office Action, because it is my opinion that the diagnostic practitioner skilled in the art would use the claimed methods as part of an overall diagnostic process, and also would not perform the claimed methods on cancerous cells. Moreover, cancer patients present with additional symptoms that are not associated with long term neurodegenerative disease (for example, unexplained weight loss, fatigue, fever, pain, youth, familial history of cancer, etc.). Such symptoms would undoubtedly help inform the

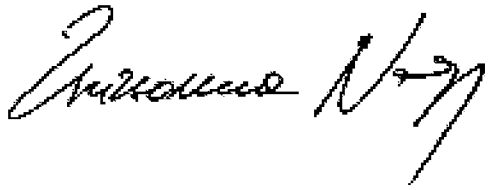
diagnostic practitioner as to the patient's true malady. Therefore, it is my opinion that a diagnostic practitioner skilled in the art would not misdiagnose a cancer patient as having a neurodegenerative disease based on the results of the claimed methods.

Ionizing And UV Radiation Induce G1 Cell Cycle Arrest

20. The Office Action states that "some of the cell cycle arrest stimuli recited in claim 6 do not arrest cells in G1, but rather G2 (i.e. ionizing and UV radiation)." Office Action at 6.

21. I respectfully disagree with the Office Action, because it is known in the art that ionizing and UV radiation do indeed arrest cells in G1. For example, Agarwal et al. noted that "[i]t is now understood more clearly that p53 mediates G1 arrest in response to DNA damage caused by UV or γ-radiation, chemotherapeutic drugs, or nucleotide deprivation." Agarwal et al., "Minireview: The p53 Network", *J. of Biol. Chem.* 273(1):1-4 (1998) at page 2; *see also* Geyer et al., "Role and Regulation of p53 during an Ultraviolet Radiation-induced G1 Cell Cycle Arrest", *Cell Growth & Differentiation* 11:149-156 (2000), which discusses G1 cell cycle arrest after exposure to ionizing and UV radiation. Copies of these references are attached hereto.

22. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Zsuzsanna Nagy, MA, MD, D.Phil.

Dated August 3, 2007.

Minireview

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The p53 Network*

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Loss of control of genomic stability is central in the development of cancer, and p53, by regulating normal responses to DNA damage and other forms of genotoxic stress, is a key element in maintaining genomic stability. Thus, it is no surprise that functional p53 is lost in about half of all human cancers. What about the other half? One possibility is that p53-independent regulatory mechanisms have been lost. Another is that inactivation of p53-dependent pathways can occur at any of several different points and that p53 itself is merely the most common target. For example, the p53 inhibitor Mdm2 is overexpressed in tumors independently of the p53 mutation. Here, we review pathways that signal *in* to p53, in response to different forms of stress, and pathways that signal *out*, triggered by activated p53. It is clear that p53 is the central component of a complex network of signaling pathways and that the other components of these pathways pose alternative targets for inactivation. For additional recent reviews, see Refs. 1 and 2.

Signaling In

The amount of p53 protein increases in response to a variety of signals, such as damaged DNA, arrest of DNA or RNA synthesis, or nucleotide depletion. The same stimuli also activate p53, which is mostly latent in the absence of stress. The increase in the amount of protein is often achieved through an increase in the half-life, from ~30 min in untreated cells to ~150 min in, for example, UV-treated cells (3). However, an increase in the rate of translational initiation of p53 mRNA can also affect the steady-state level of the protein (for example, see Ref. 4). The ubiquitin pathway probably plays an important role in degrading p53 (5), and evidence for a ubiquitin-independent mechanism of degradation has also been presented (6).

Recent evidence has also shown that the Mdm2 protein, which binds to p53, accelerates its degradation, possibly through the ubiquitin pathway (7, 8). The fact that the *mdm2* gene is a transcriptional target of p53 suggests a molecular basis for the commonly observed increased metabolic half-life of mutant p53 proteins defective in transactivation. Thus, the stability of these mutant proteins appears to be due to their inability to up-regulate the expression of Mdm2, a protein involved in their degradation, rather than an intrinsic property conferring resistance to degradation *per se*.

An increase in transactivation due to p53, with no increase in the level of the protein, was found in cells treated with low doses of UV radiation, and microinjection of an antibody to the C-terminal domain also stimulated p53-dependent transcription, even in the absence of DNA damage (9). Chernov and Stark (10) found that sodium salicylate, which inhibits protein kinases inhibits the activation of p53, with no significant effect on the accumulation of the protein. Several processes might be involved in activating p53 (1), including phosphorylation, glycosylation, binding to regulatory proteins, alternative splicing, and acetylation (11).

How does p53 sense signals? Several known proteins are suspects. The DNA-dependent protein kinase (DNAPK),[1] a plausible candidate, binds to and is activated by broken ends of DNA (12) and can phosphorylate residues 15 and 37 of p53 in a DNA-dependent manner *in vitro* (13). The phosphorylation of serine 15 affects the transactivation and growth arrest functions of p53 in some cells (14). However, cells lacking DNAPK show no defect in the p53-mediated inhibition of the cell cycle, revealing that if DNAPK has any role in regulating p53 at all, other components must be able to compensate for its loss (15).

Many protein kinases have been shown to phosphorylate p53 *in vitro* and are candidates for upstream regulators (1). However, very little *in vivo* evidence exists for the role of phosphorylation in regulating p53. Recent work showing that p53 can be acetylated *in vitro* is intriguing and suggests the possibility of an additional mechanism of regulation (11). However, it is still necessary to show that acetylation occurs in response to stress.

Poly(ADP-ribose) polymerase (PARP) has long been known to have a role in recognizing DNA damage and in DNA repair. PARP-null Chinese hamster cells are defective in activating p53 and resistant to apoptosis induced by DNA damage (16). However, embryo fibroblasts from PARP-null mice have normal DNA repair and DNA damage-induced apoptosis (17), and although there is a significant decrease in the induction of p53 protein after DNA damage or nucleotide depletion, there is no change in p53 activity or in the cellular responses to stress (18). Therefore, although PARP is involved in increasing the amount of p53 protein in mouse fibroblasts, other signaling pathways must be more important in activating p53 in response to DNA damage, consistent with experiments showing at least two levels of 53 regulation (9, 10). Loss of ATM, the product of the ataxia telangiectasia gene, slows the induction of p53 protein in response to the DNA strand breaks caused by γ-radiation but not in response to the pyrimidine dimers caused by UV radiation (19, 20). Similarly, p53 is induced normally in human *ATM*-null cells after treatment with *N*-(phosphonacetyl)-L-aspartate (PALA), which blocks *de novo* UMP biosynthesis, or adriamycin, which damages DNA.[2] p53 and ATM may both be components of complexes that function in recombination (21). Similarly, the gene product involved in Nijmegen breakage syndrome (NBS) has also been placed upstream of p53 in the pathway that responds to ionizing radiation but not in the responses to other DNA-damaging agents (22). Because the defects in p53 induction in *ATM*-null, *NBS*-null, and *PARP*-null cells are partial or selective for certain kinds of DNA

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[1] The abbreviations used are: DNAPK, DNA-dependent protein kinase; PARP, poly(ADP-ribose) polymerase; PALA, *N*-(phosphonacetyl)-L-aspartate; NBS, Nijmegen breakage syndrome; MAP, mitogen-activated protein; MAPK, MAP kinase; CAD, carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase.

[2] M. L. Agarwal and G. R. Stark, unpublished results.

damage, these gene products are involved in some but not all of the signals. Double or triple knock-outs should have a more profound (perhaps even a complete) defect in p53 induction in response to DNA damage. Similar partial defects in p53 signaling have been observed in Fanconi anemia syndrome (FAS) and Bloom's syndrome (BLS) fibroblasts, suggesting that many pathways regulate p53 (20, 23).

Recently a role for oncogenic Ras and the mitogen-activated protein (MAP) kinase pathway in p53 modulation and function has been revealed in both human and rodent cells. High expression of Ras or activation of the Mos/MAPK pathway induces wild-type p53 levels and causes a permanent growth arrest, similar to cellular senescence (24, 25). Cells lacking p53 can tolerate high levels of MAPK and display loss of p53-dependent cell cycle arrest and enhanced genomic instability (24). In a cell line defective in the MAP kinase pathway and in p53 expression, increased expression of the MAP kinase ERK2 restores the normal levels of p53, clearly placing ERK2 in a pathway that regulates the steady-state level of p53.[3] MAPK has been shown to phosphorylate residue 73 or 83 of murine p53 *in vitro,* and this phosphorylation may be important in stabilizing the protein (26). Other kinases, such as DNAPK II, cyclin A-Cdc2, and cyclin B-Cdc2, are known to phosphorylate the p53 protein *in vitro* and may play a role in stabilizing it (14, 27). The mechanisms of p53 induction in response to different types of stress are still largely unknown.

Signaling Out

p53 is involved in several different aspects of cell cycle arrest, apoptosis, control of genome integrity, and DNA repair (1, 2). How does it regulate so many different processes? p53 is a tetramer that can bind to specific sequences and thus transactivate a group of genes (reviewed in Ref. 1; for example, *p21/waf1*, *gadd45*, *mdm2*, *cyclin G*, *bax*, and *IGF-BP3*). Several groups have found that active p53 is sensed differently at different promoters, resulting in differential DNA binding and transactivation (for example, see Ref. 28). p53 can also inhibit the expression of some genes (for example, see topoisomerase IIa (29)). Furthermore, some p53-dependent phenotypes do not involve transcriptional regulation at all (for example, see Ref. 30).

Cell Cycle Controls

The G_1–S Transition—Antibodies recognizing the C terminus of p53 prevent serum-stimulated fibroblasts from entering S phase (31). This result, originally interpreted as evidence that a positive function of p53 was required, posed a paradox when overexpression of wild-type p53 was found to cause growth arrest (32). The paradox was resolved when it was found that these antibodies activate rather than inhibit p53 (9). It is now understood more clearly that p53 mediates G_1 arrest in response to DNA damage caused by UV or γ-radiation, chemotherapeutic drugs, or nucleotide deprivation (33–35). The cell-type variability in p53-dependent G_1 arrest is illustrated by studies with γ-radiation, which in normal diploid fibroblasts causes long-term, p53-dependent arrest associated with prolonged induction of p21/Waf1 (36). The irreversibility of this arrest depends on the inability of these cells to repair even a small number of double-strand DNA breaks, so that the activating signal persists (37). In contrast, γ-irradiation of HT1080 cells, derived from a fibrosarcoma with wild-type p53, causes a transient G_1 arrest (38), whereas the colorectal tumor line RKO and the breast tumor line MCF7, which also have wild-type p53, fail to arrest in G_1 following irradiation (39).

[3] M. L. Agarwal, R. Chilakamarti, W. R. Taylor, A. Agarwal, and G. R. Stark, manuscript in preparation.

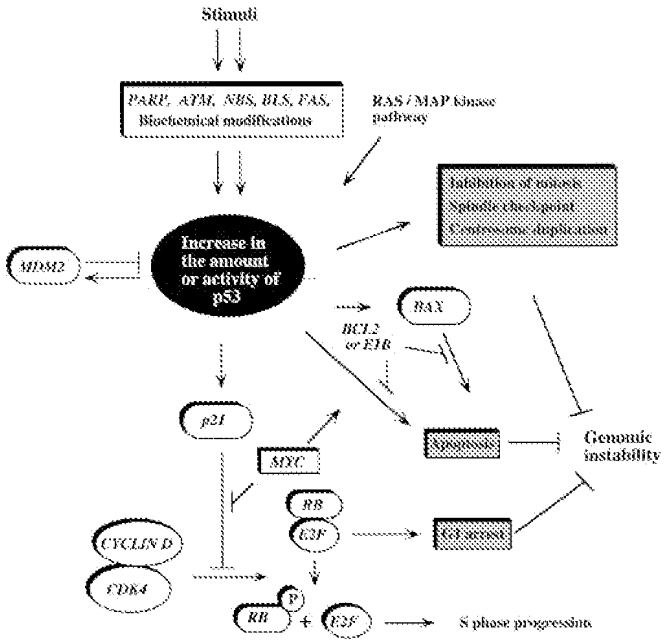

FIG. 1. **Components of p53 signaling pathways.** p53 accumulates and is modified and activated in response to signals generated by a variety of genotoxic stresses. Several proteins, including ATM, PARP, FAS, BLS, and NBS (see the text for full names), are involved in activation, but the pathways that lead to modification are largely unknown. The RAS-MAP kinase pathway is involved in establishing basal levels of p53 and may also affect function. Some of the cellular functions affected by p53 can be compromised by deregulated expression of Myc, Bcl2, E1B, or E2F. The control of p53 activity includes an autoregulatory loop involving Mdm2. The intact set of p53-dependent pathways helps to maintain genomic integrity by eliminating damaged cells, either by arresting them permanently or through apoptosis. p53 also helps to regulate entry into mitosis, spindle formation, and centrosome integrity, cell cycle checkpoints that are likely to be involved in preventing DNA damage from occurring.

These differences may indicate that tumor formation may involve the inactivation of components upstream or downstream of p53, causing the cellular response to DNA damage to fail. For example, γ-irradiation activates p53 to turn on the transcription of *p21/Waf1*, which binds to and inhibits cyclin-dependent kinases, causing hypophosphorylation of Rb, thus preventing the release of E2F and blocking the G_1–S transition (Fig. 1). Alteration of any of these downstream components may have an effect similar to that of inactivating p53 itself in preventing the pathway from functioning.

The Spindle Checkpoint—p53 is involved in a checkpoint that blocks the re-replication of DNA when the mitotic spindle has been damaged. When the DNA content of embryo fibroblasts was measured after treatment with nocodazole or other inhibitors of microtubule assembly, it was found that normal fibroblasts arrest with a 4 N content of DNA, whereas p53-null fibroblasts attain DNA contents of 8 or 16 N (40). Spindle destruction might block progression through mitosis, or re-replication might be controlled by blocking entry into S phase. In a murine cell line with wild-type p53, nocodazole causes transient mitotic arrest, followed by entry into G_1 without chromosome segregation (41). p53 is induced after mitosis is complete. The conclusion that the p53 induced in response to spindle damage blocks entry into S phase was also reached by analyzing DNA synthesis in fibroblasts exposed to nocodazole or colcemid (42). Interestingly, fibroblasts from *p21/Waf1*-null mice do not re-replicate their DNA when treated with spindle poisons, consistent with the observation that G_1 arrest in response to a number of agents is only partially abrogated in these cells (43). Therefore, p53 must also utilize p21-independ-

ent mechanisms to arrest cells in G_1 and thus to inhibit rereplication in response to spindle poisons.

Centrosome Homeostasis—Embryo fibroblasts from p53-null mice acquire more than two centrosomes, leading to mitosis with more than two spindle poles and frequent mitotic failure (44). p53 is associated with centrosomes and thus may affect centrosome duplication directly (45). Alternatively, improper duplication of centrosomes may signal p53 activation, which could in turn cause arrest in G_2 or G_1. It is intriguing that MAP kinase and Cdc2, both capable of phosphorylating p53, are also bound to centrosomes (1, 26, 46, 47) and, like p53, MAP kinase is important for centrosome homeostasis (46).

The G_2–M Transition—In both human and mouse fibroblasts, overexpression of wild-type p53 can inhibit entry into mitosis (48, 49). Recent results show that this property of p53 is important in a novel cell cycle checkpoint that controls entry into mitosis when DNA synthesis is blocked.[4] In hydroxyurea-treated cells in which synthesis of dNTPs is blocked very rapidly, p53 plays a vital role in inhibiting premature entry into mitosis. Wild-type mouse embryo fibroblasts do not attempt mitosis in hydroxyurea. In contrast, p53-null mouse embryo fibroblasts continue to attempt mitosis, entering metaphase with condensed chromatin and high levels of phosphorylated histone H1. However, cytokinesis cannot occur and mitosis is aborted, presumably because the spindles cannot segregate incompletely replicated DNA. Presumably, these responses are present to prevent the segregation of damaged or incompletely synthesized DNA.

Regulation of Apoptosis

p53 plays a role in triggering apoptosis in certain cell types, *e.g.* cells of hematopoietic origin. Stimuli such as DNA damage, withdrawal of growth factors, and expression of Myc or E1A can also cause p53-dependent apoptosis (50–54). p53 must be able to function as a transcription factor to block the G_1–S transition, but p53-mediated apoptosis does not necessarily require transcriptional activation, because inhibition of transcription by actinomycin D or translation by cycloheximide does not always affect p53-dependent apoptosis (30, 54). Furthermore, inhibitors of protein phosphatases induce p53-dependent apoptosis in the absence of transactivation (55). However, the pro-apoptotic proteins Bax and Igf-Bp3 are transcriptional targets of p53, suggesting that transactivation by p53 is important in inducing apoptosis in some circumstances. In addition, the anti-apoptotic proteins Bcl2 and the adenovirus 19-kDa E1B protein can prevent p53-mediated apoptosis (53, 56).

p53 induces apoptosis in some cell types but cell cycle arrest in others, in response to the same stimulus. Although the mechanisms of such divergent responses are not known, deletion of *p21/Waf1* can cause cells that would otherwise undergo p53-dependent cell cycle arrest to undergo apoptosis instead, underscoring the major role of genetic background in determining these cellular responses (57). Several variables, such as the extent of DNA damage and the levels of p53, also affect the choice between cell cycle arrest and apoptosis (58). Also, cross-talk between the p53 and Rb pathways may be important in determining the biological responses to DNA damage. For example, the inactivation of Rb results in the loss of G_1 arrest and induction of apoptosis after DNA damage (59). This might be explained by the release of E2F (Fig. 1), which when overexpressed on its own can induce apoptosis (60). Furthermore, overexpression of Rb blocks p53-mediated apoptosis (61). Thus, modulation of Rb and E2F through p53 signaling in response to DNA damage may play a central role in determining the balance between cell cycle arrest and apoptosis.

Genomic Stability

p53-dependent cell cycle control maintains genetic integrity in populations of cells. Gene amplification is a widely used model to study genetic integrity. In most transformed or immortalized cells, drugs such as PALA or methotrexate, which inhibit the synthesis of nucleotide precursors, select for the amplification of target genes whose products overcome the inhibition, carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase (CAD) in the case of PALA and dihydrofolate reductase for methotrexate (62). However, normal cells (63) and rare cell lines, such as REF52 (64), do not give rise to resistant colonies in these drugs. The function of p53 is lost frequently during the process of tumorigenesis (65) and in the spontaneous immortalization of primary cells (66), indicating that p53 can be the main factor determining permissivity for gene amplification. Indeed, embryo fibroblasts from p53-null mice are permissive for gene amplification (67), and primary human cells from Li-Fraumeni patients became permissive as soon as they lost their single copy of wild-type p53 (67, 68). Transformation of REF52 cells with either SV40 large T antigen or activated Ras plus E1A abolishes p53-dependent cell cycle control and allows these cells to become permissive for gene amplification (64). What signal is generated as a part of the mechanism of gene amplification that could activate p53-dependent pathways and prevent the propagation of drug-resistant cells? The current model of amplification involves, as an essential early step, multiple bridge-breakage-fusion cycles in which broken DNA is formed throughout an entire lineage of daughter cells (69, 70). The importance of DNA damage in regulating early stages of gene amplification was demonstrated with REF52 cells transfected with a temperature-sensitive mutant of SV40 large T antigen (71). When these cells are selected with PALA at low temperature, active large T antigen inactivates p53, rendering the cells permissive for gene amplification. Restoration of p53 by inactivating large T antigen at a higher temperature very early in the process of forming PALA-resistant colonies stably arrests all cells containing newly amplified DNA.

Human cell lines can achieve resistance to PALA by mechanisms other than gene amplification *in situ*, which is by far the most common mechanism in rodent cells. Most PALA-resistant colonies, from several different human cell lines, either do not contain amplified CAD DNA at all or increase the copy number of CAD as isochromosomes 2p (72). However, in both cases, p53-dependent pathways are still involved. The depletion of pyrimidine nucleotides caused by PALA generates a signal for p53 induction before any DNA damage occurs (35), arresting the cells and preventing PALA-resistant colonies from forming. Recent work has shown that overexpressing endogenous or exogenous N-Myc allows REF52 cells to overcome the p53-dependent cell cycle arrest caused by DNA damage, making these cells permissive for gene amplification (73). This observation emphasizes the fact that p53-dependent pathways can be inhibited at any of several different points (Fig. 1).

Concluding Remarks

p53 signaling pathways connect with tumor suppressors and oncogenes known to influence the cell cycle machinery (Fig. 1). Alterations in components either upstream or downstream of p53 may be analogous to inactivation of p53 itself, preventing all or a part of the entire pathway from functioning and leading to deregulation of cell cycle controls, genomic instability, and the development of cancer. In addition, the recently discovered

[4] W. R. Taylor, M. L. Agarwal, A. Agarwal, D. W. Stacey, and G. R. Stark, submitted for publication.

protein p73, which has a high degree of structural and functional homology to p53, may be another important target for inactivation during the development of cancer (74). It remains to be determined if p73 affects signals impinging on or emanating from p53 or if it is a central component of its own independent signaling network.

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Role and Regulation of p53 during an Ultraviolet Radiation-induced G_1 Cell Cycle Arrest[1]

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Abstract

p53 can play a key role in response to DNA damage by activating a G_1 cell cycle arrest. However, the importance of p53 in the cell cycle response to UV radiation is unclear. In this study, we used normal and repair-deficient cells to examine the role and regulation of p53 in response to UV radiation. A dose-dependent G_1 arrest was observed in normal and repair-deficient cells exposed to UV. Expression of HPV16-E6, or a dominant-negative p53 mutant that inactivates wild-type p53, caused cells to become resistant to this UV-induced G_1 arrest. However, a G_1 to S-phase delay was still observed after UV treatment of cells in which p53 was inactivated. These results indicate that UV can inhibit G_1 to S-phase progression through p53-dependent and independent mechanisms. Cells deficient in the repair of UV-induced DNA damage were more susceptible to a G_1 arrest after UV treatment than cells with normal repair capacity. Moreover, no G_1 arrest was observed in cells that had completed DNA repair prior to monitoring their movement from G_1 into S-phase. Finally, p53 was stabilized under conditions of a UV-induced G_1 arrest and unstable when cells had completed DNA repair and progressed from G_1 into S-phase. These results suggest that unrepaired DNA damage is the signal for the stabilization of p53, and a subsequent G_1 phase cell cycle arrest in UV-irradiated cells.

Introduction

The tumor suppressor protein p53 plays a critical role in the cellular response to DNA damage by functioning as a cell cycle checkpoint determinant (1). Wild-type p53 levels are usually quite low because of a short protein half-life (2, 3). In contrast, p53 levels increase and the protein is stabilized in response to IR,[3] and the cells undergo a G_1-phase cell cycle arrest (2–4). No G_1 arrest is observed in IR-treated cells that lack p53, indicating an essential role for p53 in the arrest response (4–6). The p53-dependent G_1 arrest is thought to allow cells time to repair the damaged DNA before proceeding into S-phase, thereby preventing an accumulation of mutations that could occur from replicating a damaged genome. Consistent with this hypothesis are reports that loss or inactivation of p53 causes cells to accumulate mutations at a higher rate (7, 8). p53 can also trigger apoptosis (programmed cell death) in certain cell types after irradiation treatment (9, 10). For example, thymocytes from p53 knockout mice were more susceptible to radiation-induced apoptosis than were thymocytes from cells expressing p53 (9). On the basis of these results and others, it has been proposed that the normal function of p53 is to monitor the integrity of the genome and protect cells from accumulating genetic damage. p53 carries out this function by temporarily halting cell proliferation to allow DNA repair or by eliminating DNA damaged cells through apoptosis.

In contrast to IR, a role for p53 in response to UV radiation has not been clarified. p53 levels increase in UV-irradiated cells as they do after IR treatment, and the cells undergo a G_1 arrest. However, in some cases this G_1 arrest was observed in normal cells and in cells in which p53 was inactivated by expression of either SV40 large T-antigen or the E6 oncoprotein of human papillomavirus (11, 12). These results suggested that the UV-induced G_1 arrest occurs in a p53-independent fashion. In contrast, a moderate G_1 arrest that appeared to be p53 dependent was observed recently in cells exposed to low doses of UV radiation (12). Furthermore, a transient G_1 arrest was observed in UV-irradiated human oral keratinocytes that expressed wild-type p53 but not in keratinocytes that lacked wild-type p53 expression (13). These results suggest that, at least in some cases, p53 can play a role in the establishment of a G_1 arrest after UV radiation treatment.

It has also been suggested that p53 may play a direct role in DNA repair after UV radiation treatment. UV radiation causes pyrimidine dimer formation and generates (6-4) photoproducts in DNA, both of which are repaired through a process called NER (14). Expression of wild-type p53 was reported to be necessary for efficient NER in UV-irradiated human fibroblasts, suggesting that p53 may play a role in the NER process (15). The best characterized NER components are the XP factors, of which there are seven, designated XP-A to XP-G. XP-B and XP-D are DNA helicases and critical

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[3] The abbreviations used are: IR, ionizing radiation; NER, nucleotide excision repair; XP, xeroderma pigmentosa; HPV, human papillomavirus; UDS, unscheduled DNA synthesis; MRD, minimum required UV dose; FCS, fluorescence-activated cell sorting.

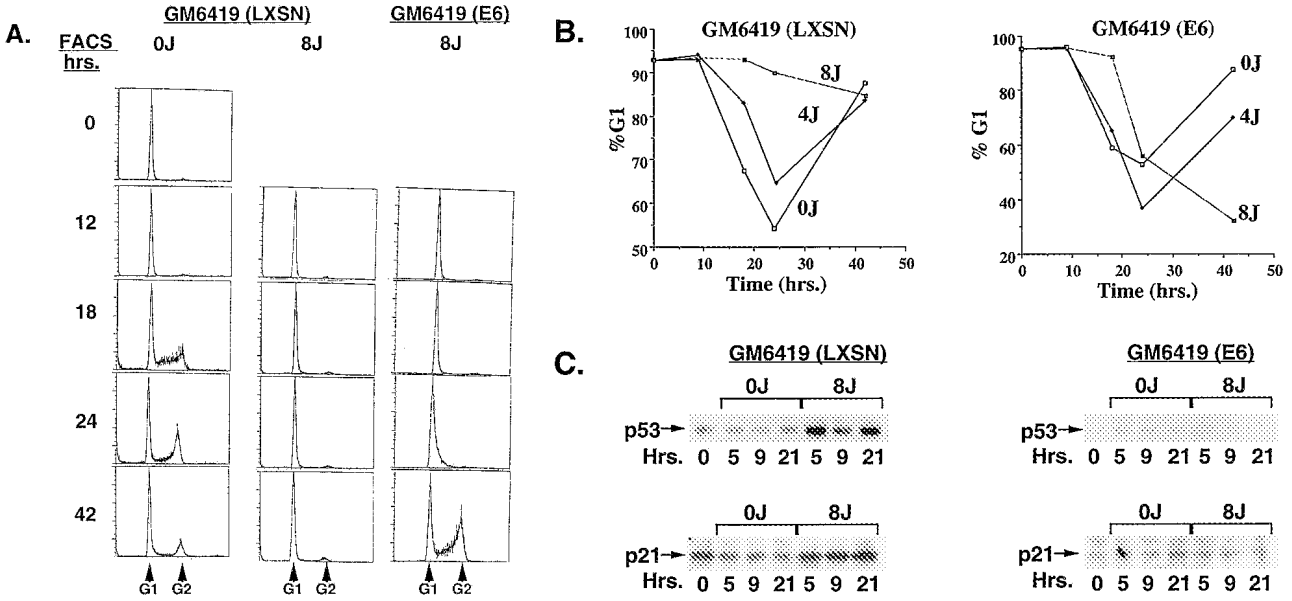

Fig. 1. GM6419 cells that were infected with a retrovirus expressing HPV-16 E6 or a control retrovirus (*LXSN*) were maintained at confluence for 48 h. The cells were then UV irradiated (0, 4, or 8 J/m^2) and plated at low density to stimulate their movement from G_1 into S-phase. Cell cycle distribution was determined by FACS analysis at various time points after plating. *A,* representative FACS data from a single experiment is illustrated and shows a complete G_1 arrest in control virus-infected cells exposed to 8 J/m^2 and a G_1 to S-phase delay in E6-expressing cells exposed to 8 J/m^2. *B,* the percentage (%) of cells with a G_1 DNA content at each time point from an experiment similar to that in *A* is plotted. The decrease in the percentage of G_1 is attributable to the movement of cells from G_1 into S-phase. *C,* cells were either nonirradiated or exposed to UV (8 J/m^2) and plated as described above. At the indicated time points after plating, protein extracts were prepared. One hundred μg of each extract were examined by Western blot analysis with the p53 antibody Ab-6 (Oncogene Science) or the p21 antibody 15431E (PharMingen).

components of the NER pathway (16, 17). p53 can interact directly with XP-B and XP-D and inhibit their helicase activities *in vitro* (18). These results raise the possibility that p53 may function during NER by modulating the activities of these two helicases. In contrast, Wang *et al.* (19) reported that XP-B and XP-D are required components of a p53-mediated apoptosis pathway (19). Therefore, the interaction between p53 and either XP-B or XP-D may mediate an apoptotic function of p53, without affecting DNA repair.

Given the role of p53 in cell cycle control and its potential role in NER, it is important to determine the relationship between UV radiation, p53, and DNA repair. In this study, we used normal and repair-deficient cell lines to examine the role and regulation of p53 in response to UV radiation. A dose-dependent G_1 cell cycle arrest was observed in normal and repair-deficient cells exposed to UV. Expression of HPV-16 E6, or a dominant-negative p53 mutant that inactivates wild-type p53, caused cells to become resistant to this UV-induced G_1 arrest. However, cells in which p53 was inactivated still underwent a significant G_1 to S-phase delay after UV exposure. These results indicate that UV can inhibit G1 to S-phase progression through p53-dependent and independent mechanisms. Repair-deficient cells were more prone to a UV-induced G_1 arrest than normal cells. Furthermore, no G_1 arrest was observed in normal cells that had completed DNA repair prior to monitoring their movement from G_1 into S-phase. Finally, p53 was stabilized under conditions of a UV-induced G_1 arrest and unstable when cells had completed DNA repair and progressed from G_1 into S-phase. These results suggest that unrepaired DNA damage is the signal for the stabilization of p53 and the subsequent p53-dependent G_1 arrest in UV-irradiated cells.

Results

Role of p53 in a UV-induced G_1 Block or Delay. The purpose of this study was to examine the role and regulation of p53 during the cell cycle response to UV radiation. Toward this end, normal human fibroblasts (GM6419 cells) were infected with control retroviruses or retroviruses that express the HPV-16 E6 oncoprotein. HPV-16 E6 promotes the rapid degradation of p53 through the ubiquitin-proteolysis pathway (20–22), and cells that express E6 are therefore similar to cells that lack p53. The effect of UV radiation on the progression of these cells from G_1 into S-phase was then assessed. Cells were maintained at confluence for 48 h to obtain G_1-phase cell populations. The cells were then treated with increasing doses of UV radiation and replated at low density to stimulate there movement from G_1 to S-phase. Progression from G_1 into S-phase was monitored by FACS analysis. As shown in Fig. 1, *A* and *B,* >90% of the cells had a G_1 DNA content at the zero time point. The percentage of nonirradiated G_1 phase cells decreased between 12 and 18 h after growth stimulation because of the movement of cells into S-phase. A UV dose of 4 J/m^2 caused a delay in the movement of control virus-infected cells into S-phase, and a UV dose of 8 J/m^2 caused a complete G_1 arrest up to 42 h after irradiation. Eight J/m^2 appeared to be the minimum dose that could cause a complete G_1 arrest in control virus-infected GM6419 cells (not shown). Cells expressing HPV-16

E6 were resistant to a UV-induced delay at 4 J/m^2, and their movement into S-phase was delayed, although not completely inhibited, at a UV dose of 8 J/m^2. p53 and p21 protein levels were also monitored in the nonirradiated and irradiated cells (Fig. 1*C*). In control virus-infected cells that were not irradiated, p53 and p21 levels were unchanged or slightly decreased after growth stimulation. In contrast, p53 and p21 levels were increased in cells treated with 8 J/m^2 prior to plating and growth stimulation. Furthermore, p53 and p21 levels were low in cells expressing HPV-16 E6, and neither p53 nor p21 were induced upon UV treatment. These results are consistent with the UV-induced arrest resulting, at least in part, from activation of the p53-p21 growth arrest pathway.

The ability of E6 to overcome a UV-induced G_1 arrest could have resulted from inactivation of p53 or from other E6 activities. To confirm the involvement of p53 in this UV-induced G_1 arrest, GM6419 cells were infected with a retrovirus encoding a dominant-negative p53 mutant (p53-CTF) capable of inactivating the wild-type p53 protein (10). The effect of UV radiation on the progression of these cells from G_1 into S-phase was then assessed (Fig. 2*A*). As with E6 expression, cells that expressed p53-CTF were resistant to a UV-induced G_1 phase arrest after exposure with 8 J/m^2 (Fig. 2*A*). These results indicate that inactivation of p53 by either the dominant-negative p53 mutant or HPV-16 E6 can overcome a UV-induced G_1 arrest. A G_1 to S-phase delay was still observed after exposure to 8 J/m^2 in p53-CTF-expressing cells, indicating that UV can also induce a G_1 to S-phase delay that is independent of p53. Steady-state levels of p53 were increased in cells expressing p53-CTF, attributable to the fact that the p53-CTF mutant can stabilize the endogenous p53 protein by sequestering it in inactive complexes (10). Nonetheless, levels of full-length p53 and p53-CTF were unchanged after UV treatment of the p53-CTF-expressing cells, and p21 protein levels were undetectable even after UV exposure (Fig. 2*B*). It should also be noted that p21 as well as MDM2 protein levels were low and not increased after IR treatment of the p53-CTF-expressing cells (not shown). Taken together, these results indicate that the p53-CTF mutant functionally inactivated the endogenous p53 protein.

p53 Mediates a UV-induced G_1 Block in UV Repair-deficient Cells. G_1 to S-phase progression was delayed in GM6419 cells exposed to 4 J/m^2 and completely blocked at a UV dose of 8 J/m^2, indicating that the extent of G_1 arrest after UV treatment was dose dependent. We predicted, based on these results, that cells deficient in the repair of UV-induced DNA damage would be more susceptible to a UV-induced G_1 arrest than normal cells. Patients with XP cannot efficiently repair UV-induced DNA damage (14, 23). XP cells from complementation group C (XPC cells) repair damage to actively transcribed DNA strands normally but are defective in the repair of nontranscribed DNA regions (24). XP cells from complementation group D (XPD cells) are defective in the repair actively transcribed DNA regions (25). XPC and XPD cells were infected with control retroviruses or retroviruses that express HPV-16 E6 or p53-CTF, and the effect of UV on their movement from G_1 to S-phase was assessed. Immunoblot analyses similar to that shown in Fig. 2 demonstrated p53-CTF expression in the XPC and XPD cells infected with the p53-CTF-expressing retrovirus (not shown). The minimum dose that caused a complete G_1 arrest up to 60 h after irradiation was ~1.5 J/m^2 in the XPD cells and 5–6 J/m^2 in XPC cells (Fig. 3). It is important to note that similar results were obtained with one other XPC and XPD cell line (not shown). Expression of either HPV-16 E6 or p53-CTF abolished the UV-induced G_1 arrest in these repair-deficient cells, indicating that the arrest was mediated in part by p53 (Fig. 3*A*). As in GM6419 cells, inactivation of p53 in these repair-deficient cells did not completely overcome the effects of UV, because a UV-induced G_1 to S-phase delay was still observed in cells expressing HPV-16 E6 or p53-CTF. Immunoblot analyses (Fig. 3*B*) indicated that p53 and p21 levels were induced by UV radiation in control cells but not induced in cells infected with either the HPV-16 E6 or p53-CTF retroviruses, consistent with the UV-induced G_1 arrest resulting in part from activation of the p53-p21 growth arrest pathway.

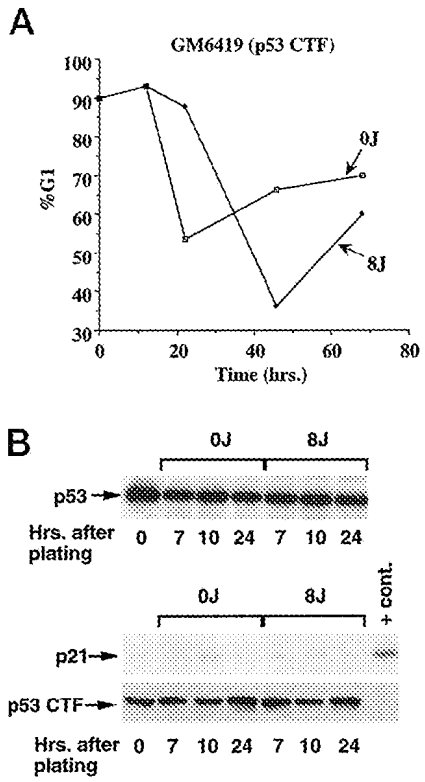

Fig. 2. A, GM6419 cells that were infected with a retrovirus expressing a dominant-negative mutant form of p53 (p53-CTF) were grown to confluence to obtain G_1-phase cells. The cells were then untreated or exposed to a UV dose of 8 J/m^2 and plated at low density to stimulate their movement from G_1 to S-phase. Cell cycle distribution was determined by FACS analysis at various time points after plating. The percentage of cells with a G_1 DNA content at each time point is indicated. *B,* cells were either nonirradiated or exposed to UV (8 J/m^2) and plated as described above. At the indicated time points after plating, protein extracts were prepared. Thirty μg of each extract were examined by immunoblotting using the p53 antibody Ab-6 for full-length p53 or the p53 antibody Ab-1 for p53-CTF, and 100 μg of extract were examined by Western blotting using the p21 antibody 15431E. The positive control for the p21 blot was 100 μg of extract from control retrovirus-infected GM6419 cells treated with 8 J/m^2 UV and harvested 21 h after plating.

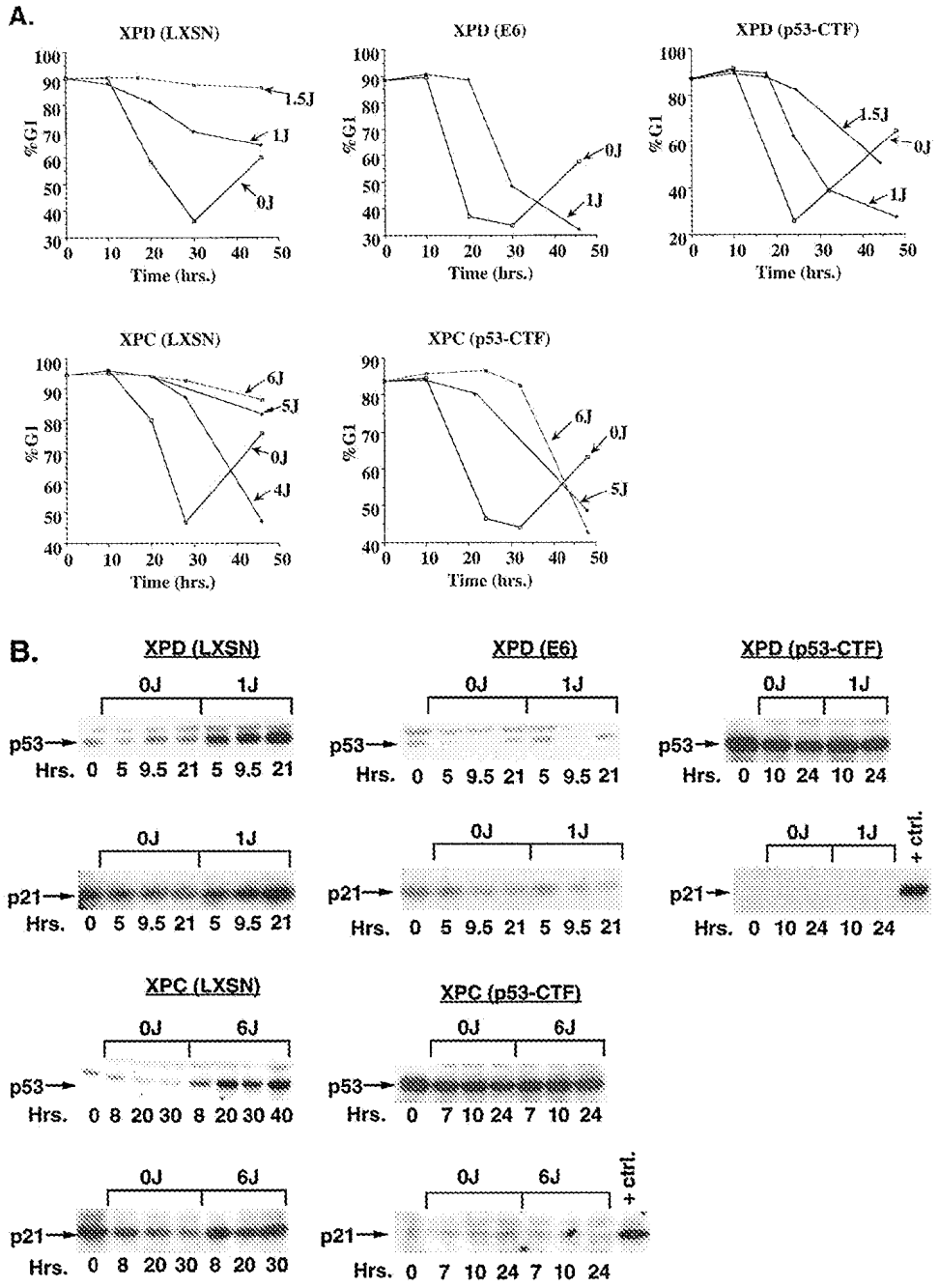

Fig. 3. A, XPC and XPD cells that were either uninfected or infected with a retrovirus expressing HPV-16 E6 or the dominant-negative mutant form of p53 (p53-CTF) were grown to confluence to obtain G_1-phase cells. The cells were then either untreated or exposed to the indicated UV dose, followed by plating at low density. Cell cycle distribution was determined by FACS analysis at various time points after plating. The percentage of cells with a G_1 DNA content at each time point is indicated. *B,* cells were either untreated or exposed to UV and plated as described above. At the indicated time points after plating, protein extracts were prepared and examined by Western blotting for p53 and p21. Thirty μg of protein extract from cells expressing the dominant-negative p53 mutant was loaded in each lane for the p53 Western blot. In all other cases, 100 μg of protein were loaded per lane. The positive control (+ *ctrl.*) for the p21 blot was 100 μg of extract from noninfected XPD or XPC cells treated with UV and harvested 21 h after plating.

Unrepaired DNA Damage Mediates a UV-induced G_1-Phase Block. Because the repair-deficient cells were more susceptible to a UV-induced arrest than normal cells, we suspected that unrepaired DNA damage may be the signal for a UV-induced arrest. To examine this possibility, cell cycle progression was analyzed in UV-irradiated cells that were first allowed to repair their DNA before being stimulated to move from G_1 into S-phase. DNA repair activity (UDS) was assessed in UV-irradiated GM6419 cells as described previously (26). Briefly, G_1 phase cells were UV irradiated and maintained in G_1 for 24 h. At various time points after UV treatment, the cells were pulse labeled with [^{3}H]thymidine. Because the cells were in G_1, the uptake of [^{3}H]thymidine was attributable to DNA repair synthesis only and not because of replicative DNA synthesis. The uptake of radionucleotide at each time point was monitored by fixing the cells directly to the culture dish and exposing them to a photographic emulsion prior to autoradiographic development. The average number of silver grains precipitated from the emulsion per cell nucleus was determined by microscopic examination and is a measure of DNA repair activity (UDS). The data are plotted in Fig. 4 as % UDS at various

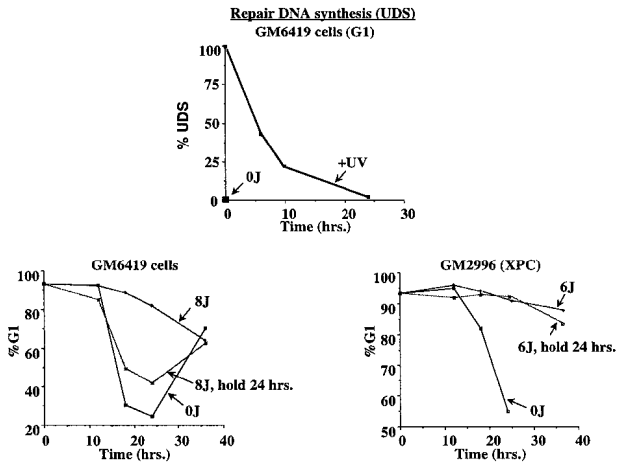

Fig. 4. Upper panel, GM6419 cells were maintained at confluence in 35-mm tissue culture dishes to obtain G_1-phase cells. The cells were then UV irradiated and maintained in the G_1 phase. At 4, 8, or 24 h after UV treatment, individual plates were incubated in the presence of 1 mCi/ml [^{3}H]thymidine for 1 h. The cells were fixed to the plate and exposed to a photographic emulsion for 1 week and then processed by autoradiography. The number of silver grains precipitated from the emulsion per cell nuclei was counted by microscopic examination and was used as a measure of repair DNA synthesis (UDS). The experiment was done in duplicate, and a minimum of 50 cells were examined on each individual plate. The highest level of repair DNA synthesis was observed immediately after UV treatment (0 time point) and was considered 100% UDS. UDS was completed after 24 h holding in G_1. *Lower panel,* G_1-phase GM6419 and GM2996 (XPC) cells were either untreated or exposed to UV doses of 8 or 6 J/m^2 as indicated. The cells were then plated at low density to stimulate their movement from G_1 to S-phase, and cell cycle distribution was determined by FACS analysis. Cells were either plated immediately after UV treatment or were held at confluence for 24 h prior to plating to allow completion of repair DNA synthesis. The GM2996 (XPC) cells show 0% UDS activity either before or after UV treatment (26).

time points after UV treatment. The level of UDS was maximal immediately after UV treatment (100% UDS) and diminished to background levels after 24 h of holding in G_1 (Fig. 4*A*). These results indicate that DNA repair after UV treatment was completed during the 24-h period that the cells were held in the G_1 phase.

Progression from G_1 into S-phase was then monitored in UV-irradiated cells that were allowed to repair their DNA prior to growth stimulation. As shown in Fig. 4*B,* GM6419 cells that were allowed to complete DNA repair prior to growth stimulation (held in G_1 for 24 h after UV treatment) were resistant to a UV-induced G_1 arrest. Furthermore, UV radiation caused a complete G_1-phase arrest in XPC cells, regardless of whether the cells were held in G_1 for 24 h prior to growth stimulation (Fig. 4*B*). These results are consistent with unrepaired DNA damage being the signal for a p53-dependent G_1 arrest in UV-irradiated cells.

Stabilization of p53 during a UV-induced G_1-Phase Block. The increase in p53 levels after UV treatment results, in large part, from stabilization of the p53 protein (2, 3). If p53 is stabilized to halt proliferation and allow DNA repair, then p53 stability is expected to decrease when DNA repair is complete. To test this possibility, p53 stability was determined in cells that were either growth stimulated immediately after UV exposure or were allowed to complete DNA repair prior to growth stimulation. The half-life of p53 was ~30 min in nonirradiated cells 12 h after release from G_1 (Fig. 5, *0J*). In contrast, p53 was stabilized (half-life extended to >2 h) in cells exposed to a UV dose of 8 J/m^2 and stimulated immediately after UV treatment. Under these conditions, the cells underwent a complete G_1-phase cell cycle arrest (Figs. 1 and 4). Importantly, the half-life of p53 was decreased to that of nonirradiated cells in cells that were UV irradiated but held in G_1 for 24 h prior to plating. Under these conditions, UV-induced DNA damage was completely repaired, and the cells progressed with normal kinetics from G_1 into S-phase (Fig. 4). These results establish an excellent correlation between p53 stability and a G_1 phase arrest in UV-irradiated cells.

Finally, p53, p21, and MDM2 protein levels were determined in UV-irradiated cells that were either growth stimulated immediately after UV exposure or were held in G_1 for 24 h prior to growth stimulation (Fig. 6). Levels of all three proteins were increased in UV-irradiated cells that were plated immediately after UV exposure and were arrested in G_1. In these experiments, p53 was induced at 5 h after release from G_1 in the UV-irradiated cells, whereas MDM2 and p21 protein levels were not increased until 10 h after release from G_1. The levels of all three proteins decreased in UV-irradiated cells that were held for 24 h in G_1 prior to plating and were resistant to the UV-induced G_1 arrest. It should be noted that p53 levels were not decreased in UV-irradiated cells held in G_1 for up to 34 h after treatment (Fig. 6), despite the fact that DNA repair was complete within 24 h of holding in G_1 (Fig. 4). This suggests that in addition to the completion of DNA repair, destabilization of p53 also requires the release of cells from the G_1 phase. The expression patterns for p53 and p21 in this experiment are consistent with the UV-induced G_1 arrest resulting from activation of the p53-p21 growth arrest pathway. It was perhaps interesting that MDM2 displayed an expression pattern similar to that of p53 and p21. MDM2 can bind p53 and promote its rapid degradation, and current models suggest that the stabilization of p53 in DNA-damaged cells results from an inhibition of p53:MDM2 binding (27–29). In Fig. 6*B,* we examined the level of p53:MDM2 binding complexes in this experiment by coimmunoprecipitation. A large amount of MDM2 immunoprecipitated with p53 from cells, which were plated immediately after UV exposure and in which p53 was stabilized. p53:MDM2 complexes were not observed until 10 h after release of the UV-irradiated cells from G_1, consistent with the increased MDM2 levels observed at this time point. The fact that p53 was stabilized with no obvious decrease in p53:MDM2 binding suggests that UV radiation may stabilize p53 through alternative pathways, in addition to inhibiting the interaction between p53 and MDM2.

Discussion

When normal mammalian cells are exposed to DNA-damaging agents, they undergo a transient G_1- and G_2-phase cell cycle arrest. These arrests allow cells time to repair the damaged DNA before proceeding with either replicative DNA synthesis or mitosis. Failure to arrest in either G_1 or G_2 phase could lead to an accumulation of mutations because of the replication of a damaged genome. IR induces a G_1 arrest in cells expressing wild-type p53 but not in cells that either lack

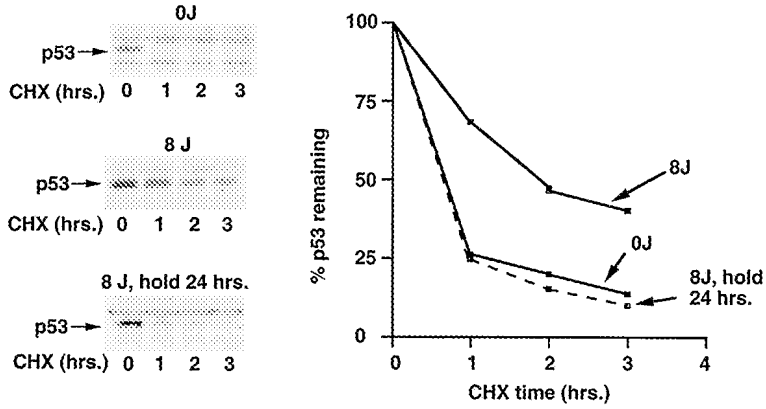

Fig. 5. GM6419 cells in the G_1-phase were either untreated (*0J*) or UV irradiated at a dose of 8 J/m^2. The cells were either plated immediately after UV treatment or were held at confluence for 24 h prior to plating. Twelve h after plating, the cells were treated with 25 μg/ml cycloheximide (*CHX*) to inhibit *de novo* protein synthesis. *Left panel,* p53 steady-state levels were monitored by immunoblot analysis at various time points after the addition of CHX. The rate at which p53 steady-state levels decline in CHX-treated cells is a measure of the protein half-life. *Right panel,* the immunoblots were quantitated on a phosphorimager. The level of p53 protein at the zero time point in each case was considered 100%, and the decrease in p53 protein levels after CHX treatment is plotted.

p53 expression or in which p53 is inactivated (4–6). These results demonstrate an essential role for p53 in the cell cycle response to IR. In contrast to IR, however, a clear role for p53 in the cell cycle response to certain other DNA-damaging agents has not been established. For example, UV radiation inhibited cell cycle progression in normal embryonic stem (ES) cells and in ES cells homozygous for a targeted deletion of p53 (30). Furthermore, high doses of either UV radiation or actinomycin D were reported to induce a G_1 arrest in cells with wild-type p53 and in cells in which p53 was inactivated by expression of the HPV-16 E6 oncoprotein (12). Finally, a p53-independent G_1 arrest was reported in murine 3T3 cells exposed to the DNA modifying agent benzo(*a*)pyrene (31). These finding indicate that certain DNA-damaging agents can signal a G_1 cell cycle arrest through mechanisms that are independent of p53.

The purpose of this study was to examine the role and regulation of p53 during a UV-induced G_1 arrest. A dose-dependent G_1 arrest was observed in normal human fibroblasts as well as in fibroblasts deficient in the repair of UV-induced DNA damage. Expression of HPV16-E6, which promotes the degradation of p53, or a dominant-negative p53 mutant that inactivates wild-type p53, caused the cells to become resistant to this UV-induced arrest. These results clearly demonstrate that p53 can activate a G_1 cell cycle arrest in response to UV radiation. Interestingly, however, cells in which p53 was inactivated still underwent a significant G_1 to S-phase delay after UV treatment. These findings indicate that UV radiation can also activate a G_1 delay that is independent of wild-type p53. On the basis of these findings, we suggest that UV radiation affects multiple pathways to cause a G_1-phase arrest or delay, only one of which involves p53.

Our results suggest that the p53-dependent G_1 arrest in UV-irradiated cells results from UV damage to actively transcribed genes. This is based on the fact that the minimum UV dose that caused a complete G_1 arrest in uninfected or control virus-infected cells was 8 J/m^2 in cells with normal DNA repair capacity (GM6419 cells), 5–6 J/m^2 in XPC cells, and 1.5–2.0 J/m^2 in XPD cells. Thus, XPD cells, which are deficient in the repair of actively transcribed genes, are more susceptible to a UV-induced G_1 arrest than are either XPC cells or normal cells, which are not compromised in the repair of transcribed DNA strands. In this regard, it is worth noting the studies of Ford and Hanawalt (15) in which wild-type p53 was required for efficient repair of nontranscribed DNA regions but not for repair of transcribed DNA strands. Insofar as p53 is not required for repair of actively transcribed genes, these results would suggest that the induction of p53 through UV damage to actively transcribed genes is independent of its role in DNA repair. Other studies support our notion that UV radiation signals to p53 through damage to actively transcribed genes. For example, the MRD that stabilized p53 was estimated in normal cells and in cells deficient in various aspects of DNA repair (32). The MRD in cells specifically deficient in the repair of actively transcribed genes was 8-fold lower than the MRD of cells with normal DNA repair capacity. In contrast, the MRD for cells specifically deficient in the repair of nontranscribed DNA regions was as high as that of normal cells. These results suggested that DNA damage to actively transcribed genes is the signal for the stabilization of p53 in response to UV radiation.

The mechanism by which UV induces a p53-independent G_1 to S-phase delay is unknown. A recent study suggested that high doses of UV radiation can inhibit the expression of E2F-1-transactivated gene products that are required for G_1 to S-phase progression (12). Thus, decreased expression of these E2F-1-regulated genes could contribute to the p53-independent G_1 to S-phase delay observed in the current report. In a separate study, UV radiation was reported to induce the expression of p21 and a concomitant G_1 arrest in Li-Fraumeni cells that lacked both *p53* alleles (33). Although this induction of p21 may explain the p53-independent responses to UV radiation in some systems, we did not observe an induction of p21 in UV-irradiated cells in which p53 was inactivated. It is worth noting that in our study, XPD cells in which p53 was inactivated by a p53 dominant-negative mutant remained more sensitive to a UV-induced G_1 arrest than either XPC or normal cells in which p53 was similarly inactivated. These results suggest that damage to actively transcribed genes may be the signal for a p53-independent G_1-phase delay, in addition to the p53-dependent arrest.

The mechanism by which UV radiation and other DNA-damaging agents stabilize p53 has not been fully clarified. MDM2 can bind p53 and promote its rapid degradation through the ubiquitin proteolysis pathway (27, 28). Current

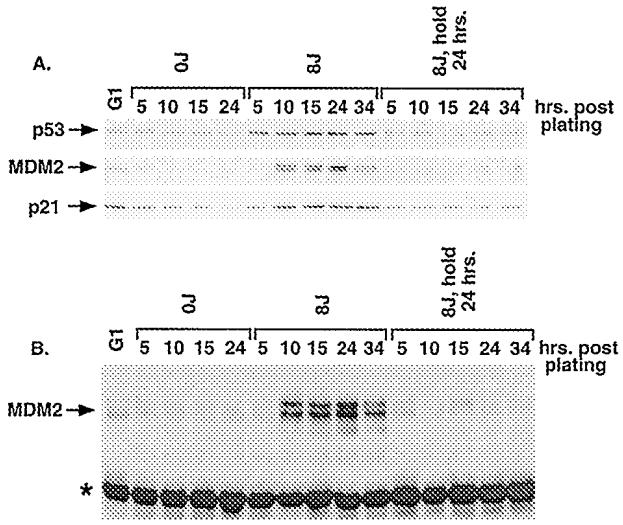

Fig. 6. A, GM6419 cells in the G_1-phase were untreated (*0J*) or UV irradiated at a dose of 8 J/m^2. The cells were either plated immediately after UV treatment or were held at confluence for 24 h prior to plating. At the indicated time points after plating, protein extracts were prepared and examined by immunoblot analysis for p53, p21, and MDM2. *B,* p53 was immunoprecipitated using the p53 antibody Ab-421 and examined by immunoblot analysis with the MDM2 antibody SMP-14 to detect p53:MDM2 binding complexes. *, position of the antibody heavy chain used in the immunoprecipitation.

models suggest that DNA-damaging agents stabilize p53 by inhibiting p53:MDM2 binding (29). According to this model, one would predict a decreased interaction between p53 and MDM2 under DNA-damaging conditions that stabilize p53. In the current study, MDM2 protein levels were increased under conditions that stabilized p53, and the UV-irradiated cells underwent a G_1 cell cycle arrest. Interestingly, the increase in MDM2 levels coincided with a corresponding increased level of p53:MDM2 binding complexes. These results raise the possibility that UV may affect multiple pathways to stabilize p53, in addition to inhibiting the interaction between p53 and MDM2.

Ineffective repair of UV-induced DNA damage can result in a high predisposition to cancer, as well as an increased sensitivity to UV-induced cell death (34, 35). Thus, efficient DNA repair after exposure to UV radiation is essential for maintaining normal cellular homeostasis. The current study indicates that UV can induce a G_1 cell cycle arrest or delay through p53-dependent and -independent mechanisms. Furthermore, our results suggest that unrepaired DNA damage to actively transcribed genes is the likely signal for a p53-dependent G_1 arrest. The presence of multiple pathways for activating a G_1 arrest or delay in response to UV radiation underlies the potential importance of such an arrest in the DNA repair response.

Materials and Methods

Cell Strains and Retroviral Infections. All cell types used in this study were maintained in DMEM containing 15% fetal bovine serum. The human diploid fibroblast strains GM6419, the XPC cell strains GM2995 and GM2996, and the XPD cell strains GM03247 and GM0524 were obtained from the Corrielle cell repository in Camden, NJ. GM6419 cells have normal repair capacity for UV-induced DNA lesions. Cell lines producing the HPV-16 E6 or control retrovirus (LXSN) were obtained from Denise Galloway (University of Washington, Seattle, WA). The DNA construct for production of the dominant-negative p53 retrovirus (referred to as p53-CTF) was obtained from Moshe Oren (Weizmann Institute of Science, Rehovot, Israel). p53-CTF encodes the COOH-terminal oligomerization domain of p53 and inactivates wild-type p53 in infected cells (10). The p53-CTF retrovirus-producing cell line was generated by Alan Thompson (Harvard Medical School). Retroviral infection was carried out by incubating exponentially growing GM6419, XPC, or XPD cells in 4 ml of medium containing a 1-ml aliquot of each retrovirus and 4 μg/ml Polybrene for 4 h. The cells were then rinsed with fresh medium once and refed with fresh medium and incubated overnight. The cells were then split at a dilution of approximately 1:4 and maintained in normal medium for an additional 24 h, at which point the cells were refed with medium containing 200 μg/ml G418. The cells were maintained in G418-containing medium for 2 weeks, and pooled populations of selected cells were obtained.

UV Radiation Treatment and Cell Cycle Analysis. UV irradiation was carried out as described previously (2). The UV light exposure apparatus consisted of five UV bulbs in a specially constructed incubator that delivered 254 nm light at a dose of 2.08 J/m^2/s. Confluent, G_1-phase cells were rinsed with PBS and exposed to the indicated UV dose. The cells were then trypsinized and replated at low density to stimulate their movement from G_1 into S-phase. At the indicated time after growth stimulation, cells were trypsinized and fixed in 70% ethanol. The fixed cells were suspended in PBS containing 1 mg/ml propidium iodide and 1000 Kunitz units/ml RNase A. Cell cycle distribution was determined by FACS analysis at the Dana-Farber Flow Cytometry Laboratory.

Western Blots, Immunoprecipitations, and p53 Stability Measurements. For Western blot analysis, cells were washed twice with PBS, scraped into 0.5 ml of lysis buffer [50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, and 1 mM phenylmethylsulfonyl fluoride], and incubated on ice for 15 min with occasional light vortexing. Lysates were spun at 15,000 × *g* for 15 min to remove cellular debris. Protein extract from the resulting supernatant was resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) for detection with either the Ab-6 p53 antibody (Oncogene Science), the anti-p21 polyclonal antibody 15431E (PharMingen), or the anti-MDM2 antibody SMP-14. For analysis of p53:MDM2 binding, p53 was immunoprecipitated from lysates using the p53 antibody Ab-421 (Oncogene Science) and subsequently examined by immunoblot analysis using the MDM2 antibody SMP-14.

DNA Repair Measurements. DNA repair activity (UDS) was assessed as described (26) in UV-irradiated GM6419 cells in the following manner. G_1 phase cells were UV irradiated (8 J/m^2) and maintained in G_1 for 24 h. At various time points after UV treatment, the cells were pulse labeled with 10 μCi [^{3}H]thymidine. Because the cells were in G_1, the uptake of [^{3}H]thymidine in the general cell population was attributable to DNA repair synthesis only and not attributable to replicative DNA synthesis. The uptake of radionucleotide at each time point was monitored by fixing the cells directly to the culture dish and subsequently exposing them to a photographic emulsion prior to autoradiographic development. The average number of silver grains precipitated from the emulsion per cell nucleus was determined by microscopic examination and was used as a measurement of DNA repair activity. The experiment was performed in duplicate, and the average number of silver grains precipitated per cell nucleus at each time point was determined. The highest number of silver grains were precipitated from each cell nucleus immediately after UV treatment.

Acknowledgments

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